*Journal of Bioenergetics and Biomembranes, Vol. 23, No. 2, 1991*

# MINI-REVIEW

# Cytochrome *c* Oxidase in *Paracoccus denitrificans*. Protein, Chemical, Structural, and Evolutionary Aspects

Gerhard Buse[1] and Guy C. M. Steffens[1]

*Received August 28, 1990*


## Abstract

Preparations and protein chemical characterizations performed with cytochrome *c* oxidase (E.C. 1.9.3.1) from the purple bacterium *Paracoccus denitrificans* are reviewed. The simplest catalytically competent complex of the enzyme consists of two subunits of 62012 and 27999 Da. The theoretical heme *a*/protein ratio of the purified enzyme is 22.0 nmol/mg. The amino acid sequences of both proteins are compared with examples of subunits I and II of mitochondrial terminal oxidases from the main kingdoms of eukaryotes. The significance of the emerging conserved features such as membrane penetration patterns, invariant residues, stoichiometry, and sites of prosthetic groups are discussed. The *Paracoccus* enzyme represents the only prokaryotic oxidase detailed so far, which is directly related to the mitochondrial oxidases by common ancestry in the growing $O_2$ atmosphere.


**Key Words:** Cytochrome *c* oxidase; membrane protein preparation; endosymbiosis; purple bacteria; mitochondrial origin; metal analysis; protein–metal stoichiometry; $O_2$ atmosphere; 16s rRNA phylogeny.

## Introduction

At the beginning of the century, discoverers of mitochondria recognized their histological similarity with bacteria (Altmann, 1890). These microscopists thus started a series of descriptions which later were carried on by biochemists, unraveling the self-contained inner membrane (cardiolipin), the prokaryotic type of protein biosynthesis (small ribosomes, formyl methionine,

[1]Institut für Biochemie, Rheinisch-Westfälische Technische Hochschule Aachen, Klinikum Pauwelsstr. 30, D-5100 Aachen, Germany.



0145-479X/91/0400-0269$06.50/0 

circular DNA) of this organelle, finally reaching the field of molecular genetics with the description of the systematic position of mitochondria by comparative sequence analysis of their 16s rRNA as prokaryote-like symbionts derived from the α-group of purple bacteria (Woese, 1987). This evidence then means that bacteria of this group should best resemble the biochemical heritage which mitochondria introduced into the eukaryotic cell.

Mitochondria are the site of cellular respiration and energy conservation, and this central biochemical function of the eukaryotic cells may therefore *in nuce* be seen in nonphotosynthetic purple bacteria. *Paracoccus denitrificans* is among this group. Catalysis of the reaction

$$4\text{Cyt } c^{2+} + 8H_i^+ + O_2 = 4\text{Cyt } c^{3+} + 4H_0^+ + 2H_2O$$

by cytochrome *c* oxidase (EC 1.9.3.1) may in essence be studied with the *Paracoccus* enzyme (Azzi *et al.*, 1985).

Another aspect coming into play with these bacteria is that photosynthesis is still active in several species of this group. The photosynthetic reaction center, the only complex membrane proteins so far successfully crystallized and solved by X-ray analysis (Deisenhofer *et al.*, 1985), has been obtained from *Rhodopseudomonas viridis* while other lines of these bacteria, among them *Paracoccus*, have lost this capability. Nevertheless the existing relationship between photosynthesis and respiration may with advantage be studied here. By analogy, cytochrome *c* oxidase prepared from *Paracoccus denitrificans* seems to be a good choice for the successful 3d-crystallization and X-ray analysis of this important enzyme.

As had already been suggested (Buse *et al.*, 1983), after discovery of the relatively close relationship and functional similarity at the first isolation of the terminal oxidase, the two-subunit enzyme (Ludwig and Schatz, 1980), there exists also the genetic information homologous to subunit III of mitochondrial enzymes in this organism and, depending on the preparation (Raitio *et al.*, 1987), the enzymatic function may be recognized in different protein aggregation states, among them the three-subunit oxidase (Haltia *et al.*, 1988).

The protein chemical outlines given in this article, however, mainly refer to the most simple form of the catalytically competent enzyme isolated from this bacterium, the two-subunit oxidase.

## Preparation of Cytochrome *c* Oxidase from *Paracoccus denitrificans*

The first preparation of cytochrome *c* oxidase from *Paracoccus denitrificans* was described by Ludwig and Schatz (1980). Their preparation protocol started with the disruption of the cells, sedimentation of a

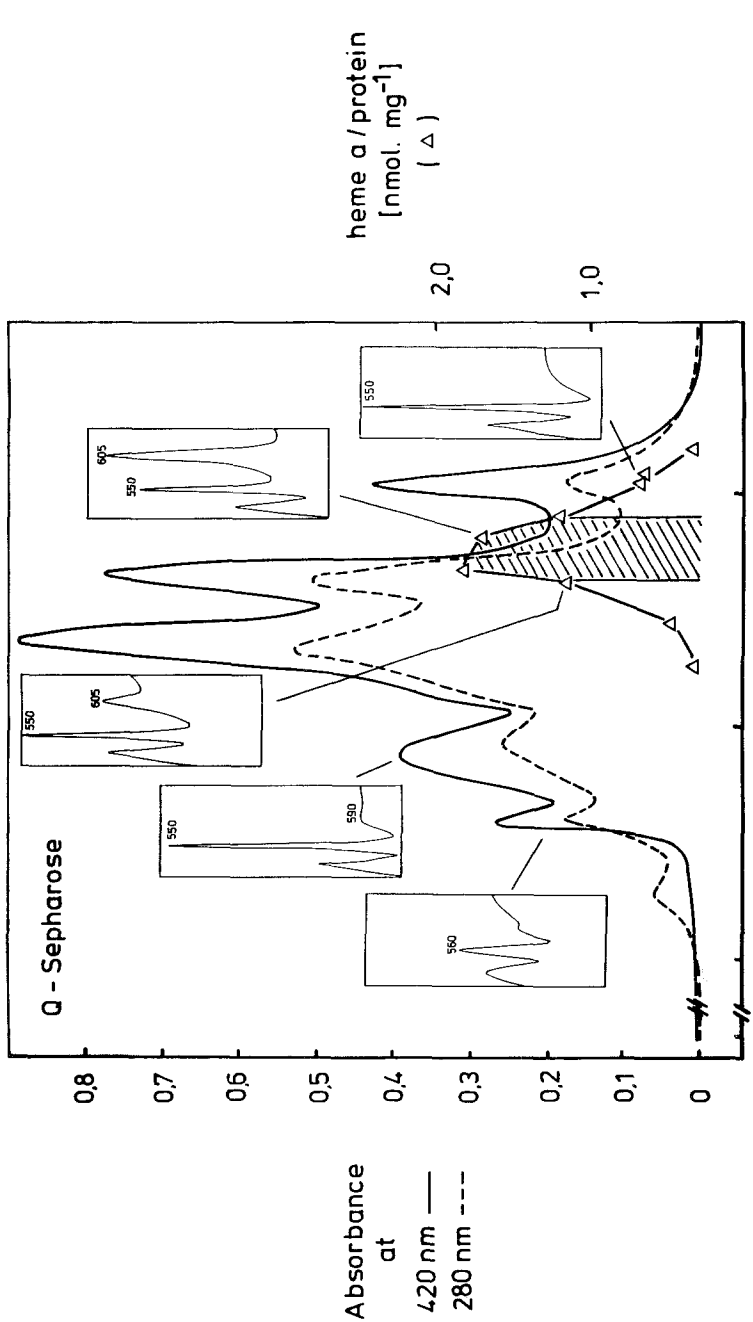


**Fig. 1.** Ion-exchange chromatography of crude cytochrome *c* oxidase on Q-Sepharose Fast Flow (20 × 5 cm) in 10 mM potassium phosphate (pH 7.8); 1% TX-100 and 0.1 mM EDTA. Proteins were eluted with a linear gradient from 0–1 M NaCl in the same buffer. Inserts show reduced-minus-oxidized difference spectra of various heme protein fractions.

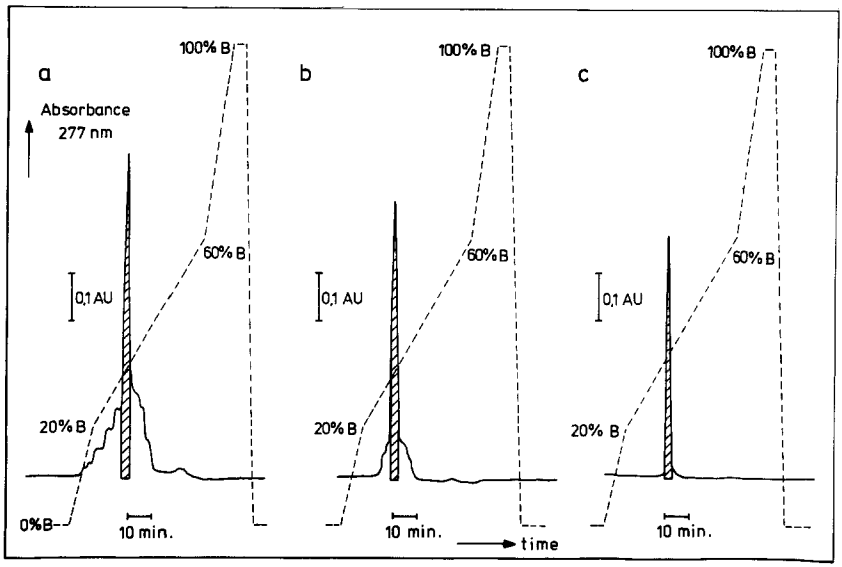


**Fig. 2.** Series of consecutive chromatographic steps on Mono Q. In experiment a, the crude product from Q-Sepharose with an *h/p* of 1.5 nmol/mg is further purified. The material corresponding to the shaded area was used for further purification and/or final characterization.

membrane-rich fraction, subsequent washing of the crude membranes with deoxycholate, and solubilization of the cytoplasmic membranes with Triton X-100 (TX-100). By fractionated ammonium sulfate precipitation and ion-exchange as well as gel-filtration chromatography the enzyme was purified to homogeneity of the protein components. Polyacrylamide gel electrophoresis revealed a clear two-subunit structure. The apparent molecular masses were estimated to 45,000 and 28,000 (Ludwig and Schatz, 1980). The enzyme was suggested to be a monomer under these conditions (Ludwig *et al.*, 1982). The overall yield of this preparative procedure was about 11%, similar to that obtained in preparations of cytochrome *c* oxidases from other bacterial sources (Fukumori *et al.*, 1985; Sone and Yanagita, 1982; Yoshida *et al.*, 1984). This rather low yield, and the nevertheless elaborate isolation procedure, prompted Steffens *et al.* (1987) to improve the method by introducing newly developed separation matrices and recently published techniques. The initial steps of the procedure, i.e., homogenization, treatment with deoxycholate, and solubilization with TX-100, were not changed. The next step is either a gel-filtration chromatography on Ultrogel AcA 34 or ion-exchange on Q-Sepharose. Although these steps are characterized by relatively low yields and low purification rates (Table I) they are important since they remove highly aggregated protein and phospholipids. The material, which at this stage in most cases has a heme a/protein ratio of about


**Fig. 3.** Polyacrylamide gel electrophoresis of the two-subunit preparation of cytochrome *c* oxidase from *Paracoccus denitrificans* (lanes 2,3). For comparison the multi-subunit bovine cytochrome *c* oxidase is shown (lanes 1,4).

0.8 nmol/mg, is then applied to a Q-Sepharose Fast Flow column (Pharmacia LKB). The various proteins are eluted by a linear gradient (described in Fig. 1) or by stepwise elution with buffers of increasing NaCl molarities. Several hemeproteins can be identified by recording reduced-minus-oxidized different spectra. Thus cytochrome *b* or cytochrome *o* (*bo*) (560 nm), cytochrome $a_1$ (590 nm), cytochrome $aa_3$ (604 nm), as well as several cytochromes *c* (Fig. 1) (550–552 nm) are observed. The fractions with the highest heme *a*/protein ratios are pooled and used for the final purification steps by fast protein liquid chromatography on Mono Q (Pharmacia LKB). Here purification rates of 3–4 with repetitive yields of 80–90% are routinely achieved (Table I and Fig. 2). Two to three consecutive chromatographies, which can

**Table I.** Preparation of Cytochrome *c* Oxidase from *Paracoccus denitrificans* by FPLC on Mono Q

| | Heme *a* (nmol)[a] | Protein (mg) | Heme *a*/protein (nmol/mg) | Yield[b] (%) | Overall yield (%) |
|---|---|---|---|---|---|
| TX-100 membrane extract | 2.100 | 6.550 | 0.3 | 100 | 100 |
| Ultrogel AcA 34 | 1.200 | 1.714 | 0.7 | 55 | 55 |
| Q-Sepharose | 715 | 477 | 1.5 | 60 | 36 |
| Mono Q Run a | 587[c] | 129[c] | 4.6 | 82 | 28 |
| Mono Q Run b | 529 | 41 | 13.0 | 90 | 25 |
| Mono Q Run c | 467 | 22.9 | 21.9 | 88 | 22 |

[a] 2 nmol heme *a* correspond to 1 nmol cytochrome *c* oxidase.
[b] Yield in percentage of heme *a* of TX-100 membrane extract.
[c] Since the binding capacity of Mono Q HR 10/10 is limited, only ca. 100 mg portions are used for further purifications. The data refer to the sum of five runs.

be performed in the course of one day, generally lead to pure two-subunit cytochrome *c* oxidase as demonstrated by polyacrylamide gel electrophoresis (Fig. 3) and a heme *a*/protein ratio close to the theoretical value of 22.0 nmol/mg (Table I). An overall yield (Fig. 3) of about 22% can be obtained with a substantial saving of time (Steffens *et al.*, 1990).

## Characterization of Cytochrome *c* Oxidase from *Paracoccus denitrificans*

### *The Proteins*

As mentioned in the introduction, preparations of cytochrome *c* oxidase depending on the preparation protocol and on the nature of the detergents used (Haltia *et al.*, 1988), are characterized as two- or three-subunit complexes or as a so-called supercomplex (Berry and Trumpower, 1985). Since the two-subunit cytochrome *c* oxidase was the first complex to be investigated and clearly is more stable and thus more suited for the study of structure/function relationships and appears to be fully enzymatically competent in terms of the hitherto known functions of the enzyme, most studies have been undertaken with the two-subunit complex. All data dealt with in this section apply to this state. Table II gives the amino acid analyses and compositions of the complex two-subunit cytochrome *c* oxidase and its isolated subunits. The analysis of the complete enzyme is compared with the sum of the composition of the individual subunits deduced from their respective gene structures. In the case of subunit II, the composition of the mature protein is used (see below and Table II).

The two subunits were separated by gel-filtration chromatography on Bio-Gel P100 in 3% SDS as described earlier (Steffens and Buse, 1976). Aliquots of both subunits were, after reductive carboxymethylation, subjected to total hydrolysis and subsequent amino acid analysis.

Both polypeptides were shown to be N-terminally blocked in Edman degradation experiments (Steffens *et al.*, 1983). The proteins were further characterized by sequencing of cyanogen bromide and tryptic fragments. The homologous alignments of the partial sequences to mitochondrial cytochrome *c* oxidases supported the common origin of bacterial and mitochondrial cytochrome *c* oxidase and thus of bacteria and mitochondria at a molecular level (Steffens *et al.*, 1983). In the course of the protein chemical studies with subunit II, both the N- and C-terminal tryptic fragments were isolated. The N-terminal fragment was subjected to fast atom bombardment (FAB) mass-spectrometric analysis by which the N-terminal blocking group was identified as a pyroglutamic acid residue (Steinrücke *et al.*, 1987). The C-terminus was characterized by treatment with carboxypeptidase A, which

**Table II.** Amino Acid Composition and Analyses of Cytochrome *c* Oxidase and Its Constituting Subunits from *Paracoccus denitrificans*

| | | | | | Subunit II | | |
|---|---|---|---|---|---|---|---|
| | $aa_3$-Oxidase | | Subunit I | | | DNA sequence | |
| Amino Acid | AAA | DNA sequences[a] | AAA | DNA sequence[b] | AAA | 29-280[c] | 1-297[d] |
| Cys | n.d. | 7 | 3.0 | 3 | 1.8 | 4 | 4 |
| Asx | 58.1 | 53 | 39.9 | 30 | 24.1 | 23 | 25 |
| Asp | | 29 | | 15 | | 14 | 16 |
| Asn | | 24 | | 15 | | 9 | 9 |
| Thr | 42.2 | 43 | 38.2 | 34 | 9.9 | 9 | 12 |
| Ser | 36.3 | 38 | 27.7 | 29 | 8.9 | 9 | 13 |
| Glx | 59.3 | 55 | 31.6 | 30 | 26.2 | 25 | 26 |
| Glu | | 28 | | 15 | | 13 | 14 |
| Gln | | 27 | | 15 | | 12 | 12 |
| Pro | n.d. | 50 | 31.6 | 33 | 17.1 | 17 | 20 |
| Gly | 66.4 | 65 | 64.4 | 51 | 15.2 | 13 | 16 |
| Ala | 81.5 | 77 | 54.9 | 50 | 25.5 | 28 | 39 |
| Val | 68.3 | 66 | 35.5 | 37 | 27.2 | 28 | 34 |
| Met | 30.2 | 34 | 25.5 | 30 | 4.3 | 4 | 7 |
| Ile | 56.7 | 58 | 31.1 | 40 | 16.2 | 18 | 19 |
| Leu | 75.1 | 77 | 54.4 | 53 | 23.8 | 24 | 28 |
| Tyr | 29.6 | 36 | 24.4 | 27 | 8.3 | 9 | 10 |
| Phe | 54.2 | 58 | 43.8 | 46 | 12.4 | 12 | 12 |
| His | 19.8 | 25 | 23.2 | 19 | 8.6 | 6 | 6 |
| Lys | 22.5 | 18 | 12.2 | 9 | 8.3 | 9 | 11 |
| Arg | 23.8 | 21 | 16.6 | 15 | 7.0 | 6 | 8 |
| Trp | n.d. | 25 | n.d. | 18 | n.d. | 6 | 8 |
| Total | | 806 | | 554 | | 252 | 297 |
| $M_r$ | | 90.817 | | 62.012 | | 27.999 | |

[a]Sum of compositions of subunits I and II, see footnotes *b* and *c*.
[b]Composition of the complete translated gene (Raitio *et al.*, 1987).
[c]Composition of the mature protein (Steinrücke *et al.*, 1987).
[d]Composition of the translated gene (Steinrücke *et al.*, 1987).

identified two alanines and one phenylalanine as the C-terminal amino acids. The final proof was obtained from the isolation of the C-terminal tryptic fragment with the sequence -Glu-Glu-Phe-Ala-Ala (Steinrücke *et al.*, 1987). Thus, the mature subunit II is initially synthesized as a longer precursor with 297 amino acid residues. After processing in two steps by (specific) protease(s) and post-translational modification of the new N-terminal glutamine into a pyroglutamic derivative, the mature protein with 252 residues is formed (see Fig. 5). The sequence at the N-terminal processing site has some characteristics in common with signal sequences around processing sites in eukaryotic proteins (von Heijne, 1985). Also the C-terminal processing may be the result of a distinct proteolytic step since the cytochrome *c* 550 and the mature subunit II of cytochrome *c* oxidase from *Paracoccus denitrificans* both have identical C-termini: -Ala-Ala (Ambler *et al.*, 1981). Since subunit I is also blocked by a hitherto undetermined group—possibly also pyroglutamic acid—it is tempting to speculate that subunit I has also been processed and post-translationally modified. The glutamine in position 4, which is preceded by an alanine, might be a candidate on the basis of similarity to the identified processing sites in subunit II and cytochrome *c* 550. With the exception of Asx and Gly, a fairly good agreement is observed between the amino acid analysis of subunit I (Table II) and the composition deduced from the gene (Raitio *et al.*, 1987). Thus, it is likely that processing of this chain removes only relatively small N-terminal and/or C-terminal parts if any. The overall hydrophobicity calculated according to Kyte and Doolittle (1982) is 0.62 for subunit I and 0.35 for subunit II as compared to 0.73 and 0.22 respectively, for the bovine proteins.

### *The Metals*

In bovine heart cytochrome *c* oxidase the number of metals has been a matter of controversy for a long period (Van Gelder and Beinert, 1969). The observation of the stoichiometric presence of zinc and magnesium in the bovine heart protein by Einarsdóttir and Caughey (1984, 1985) using inductively coupled plasma atomic emission spectroscopy has initiated a series of (re)investigations of the metal stoichiometries in cytochrome *c* oxidases from various species. In the course of these studies Steffens *et al.* (1987) could show that cytochrome *c* oxidase from bovine heart, *Paracoccus denitrificans*, and *Dictyostelium discoideum* are characterized by the presence of three copper atoms per two hemes *a*. Meanwhile they have investigated a larger number of preparations of both bovine heart and *Paracoccus denitrificans* cytochrome *c* oxidase. The results of these analyses are compiled in Table III.

The observation of three stoichiometric copper ions per monomer of cytochrome *c* oxidases is clearly confirmed for both species. Both calculation

**Table III.** Metal Stoichiometries (A) and Heme *a*/Protein Ratios (B) of Preparations of Cytochrome *c* Oxidase from *Paracoccus denitrificans* and Bovine Heart as Determined by ICP-AES

| | *Paracoccus denitrificans* | | Bovine heart | |
|---|---|---|---|---|
| Metal/element | Stoichiometries based on mutual ratios[a] | Stoichiometries based on simultaneous sulfur determination[b] | Stoichiometries based on mutual ratios[c] | Stoichiometries based on simultaneous sulfur determination[d] |
| | | A | | |
| Cu | 2.98 ± 0.25 (14)[e] | 3.01 ± 0.49 (8) | 2.87 ± 0.10 (17) | 2.88 ± 0.33 (12) |
| Fe | 2.02 ± 0.25 (14) | 1.91 ± 0.16 (8) | 1.99 ± 0.10 (17) | 2.05 ± 0.18 (12) |
| Zn | 0.64 ± 0.37 (14) | 0.48 ± 0.23 (8) | 1.11 ± 0.13 (17) | 1.15 ± 0.22 (12) |
| Mg | 1.24 ± 0.49 (10) | 1.05 ± 0.47 (7) | 1.03 ± 0.06 (15) | 1.02 ± 0.18 (10) |
| Mn | 0.19 ± 0.04 (14) | 0.18 ± 0.04 (8) | 0.008 ± 0.006 (17) | 0.008 ± 0.007 (9) |
| P | 3.30 ± 2.37 (9) | 1.73 ± 0.80 (5) | 22.62 ± 7.00 (10) | 21.98 ± 4.48 (8) |
| S | 41.12 ± 3.91 (9) | 41 | 95.33 ± 10.82 (16) | 94 |
| | | B | | |
| | Heme *a*/protein ratios | *Paracoccus denitrificans* (nmol/mg) | Bovine heart (nmol/mg) | |
| | Experimental values: | | | |
| | spectrophotometric with *E* 12000 | 23.2 | 10.47 | |
| | iron determination by ICP-AES | 22.5 | 9.77 | |
| | Theoretical value | 22.0[f] | 9.79[g] | |

[a] $[aa_3] = [Cu] + [Fe]/5$.
[b] $[aa_3] = [S]/41$.
[c] $[aa_3] = [Cu] + [Fe] + [Zn] + [Mg]/7$.
[d] $[aa_3] = [S]/94$.
[e] Number of preparations in parentheses.
[f] $M_r = 90817$ (see Table III).
[g] $M_r = 204266$.

procedures essentially lead to the same results. Protein-correlated stoichiometries are directly obtained on the basis of the simultaneously determined sulfur, which results from the protein-incorporated sulfur amino acids cysteine and methionine. For both enzymes the data convincingly demonstrate that monomers of the bovine heart oxidase with 94 sulfur atoms and of *Paracoccus denitrificans* oxidase with 41 sulfur atoms contain three coppers. The observation of a third copper ion does not necessarily imply its redox activity. As a matter of fact, cytochrome *c* oxidase from bovine heart only accepts four electrons upon transition from the fully oxidized to the fully reduced state, and vice versa (Steffens and Buse, 1990; Schroedl and Hartzell, 1977; Brunori *et al.*, 1979). The recent proposal of Kroneck *et al.* (1988) for a binuclear copper center at the $Cu_A$ site fits to both the observation of a third copper ion and cytochrome *c* oxidase being a four-electron acceptor. On the other hand, the above data on the excellent correlation of the metal and sulfur stoichiometries support the subunit stoichiometries proposed for bovine heart (subunits I–XIII present in a 1 : 1 ratio) Buse *et al.*, 1985; Verheul *et al.*, 1981; Merle and Kadenbach, 1980) and *Paracoccus denitrificans* (subunits I–II present in a 1 : 1 ratio) (Ludwig and Schatz, 1980), i.e., these investigations for the first time related copper to the exact protein $M_r$ instead of heme *a*.

The data of Table III also confirm the stoichiometric presence of zinc and magnesium in the bovine protein. In the case of the bacterial enzyme, however, these metals are not present in integral amounts. Both the zinc and magnesium contents vary considerably from preparation to preparation. Zinc clearly is substoichiometric, whereas the magnesium stoichiometry in many preparations exceeds unity. Its deviation from this value (standard deviation $\pm$ 0.5) leaves doubt about its stoichiometry being integral; more data on the magnesium stoichiometries also in other bacterial cytochromes *c* oxidases, e.g., *Rhodopseudomonas*, are necessary to overcome this uncertainty. The presence of substoichiometric amounts of manganese has been observed earlier and makes the EPR investigation of the copper signals of this enzyme difficult. Only a substantial reduction of manganese in the culture medium reduces the manganese concentration in the cytochrome *c* oxidase preparations and enables EPR spectra determination (Seelig *et al.*, 1981). It is clear from the latter data that the preparations of the *Paracoccus* enzymes obtained so far are not strictly homogeneous.

### *The Phospholipids*

The number of phospholipids bound to cytochrome *c* oxidase from *Paracoccus denitrificans* has also been determined indirectly by inductively coupled plasma atomic emission spectroscopy (Steffens *et al.*, 1987). In these

experiments the number of phosphorus atoms per monomer (i.e., per 41 sulfur atoms of per 2 heme irons) was estimated to vary from 1–5 (Table III). Thus, preparations of cytochrome *c* oxidase from *Paracoccus denitrificans* prepared by FPLC in TX-100 are characterized by a relatively low phospholipid content. Bovine heart cytochrome *c* oxidase, prepared according to the authors' method (Steffens and Buse, 1976) for comparison, binds about 21 phosphorus atoms per monomer, which, depending on the nature of the phospholipids, means 10–20 molecules. At present no information is available on the nature of the phospholipids bound to cytochrome *c* oxidase from *Paracoccus denitrificans*, except that the plasma membrane of *Paracoccus* has phosphatidylcholine as the main constituent with straight-chain fatty acids of saturated and unsaturated type and no cholesterol (John and Whatley, 1977).

## Alignment of Sequences (Homology)

Figure 4 shows the computer alignment of subunit I sequences including one species each of the main kingdoms of eukaryotes—animals, plants, and fungi—compared with the *Paracoccus* sequence. It should be noted that this entire sequence information has primarily been obtained from DNA sequencing; there is no protein proof for these sequences with the exception of the bovine sequence which has recently been completed in the author's laboratory (Hensel and Buse, 1990) giving the only protein evidence for the subunit I structures of so far about 20 DNA-derived sequences. Correspondingly, only this sequence can be considered fully established while some questions remain with the others including the *Paracoccus* subunit I. Among these are: In the original paper by Bonitz *et al.* (1980) on the yeast subunit I, positions of introns have been deduced from the then available human sequence (Anderson *et al.*, 1981) which according to the extremely dense construction of mammalian mtDNA has no intervening sequences. Erroneous positioning has, however, been noticed, which is especially important for the persistence of histidine 378 for tyrosine in an invariant position of the yeast protein (Protein Information Resources, 1989). It was further not recognized in the early papers that, also in yeast, ATA of the mtDNA codes for methionine 87 instead of isoleucine.

In the wheat sequence (and plant sequences in general) caution is necessary because of the so far not well-understood phenomenon of "editing" of RNA messengers (Jukes, 1990; see also wheat subunit II) whereby cytosine is changed to uracil at several codons. No evidence has been presented for positioning of the terminal sequences or absence of site-specific modification from the protein. Introns have not been discussed in this analysis (Covello and Gray, 1989). There exists also no protein chemical corroboration so far

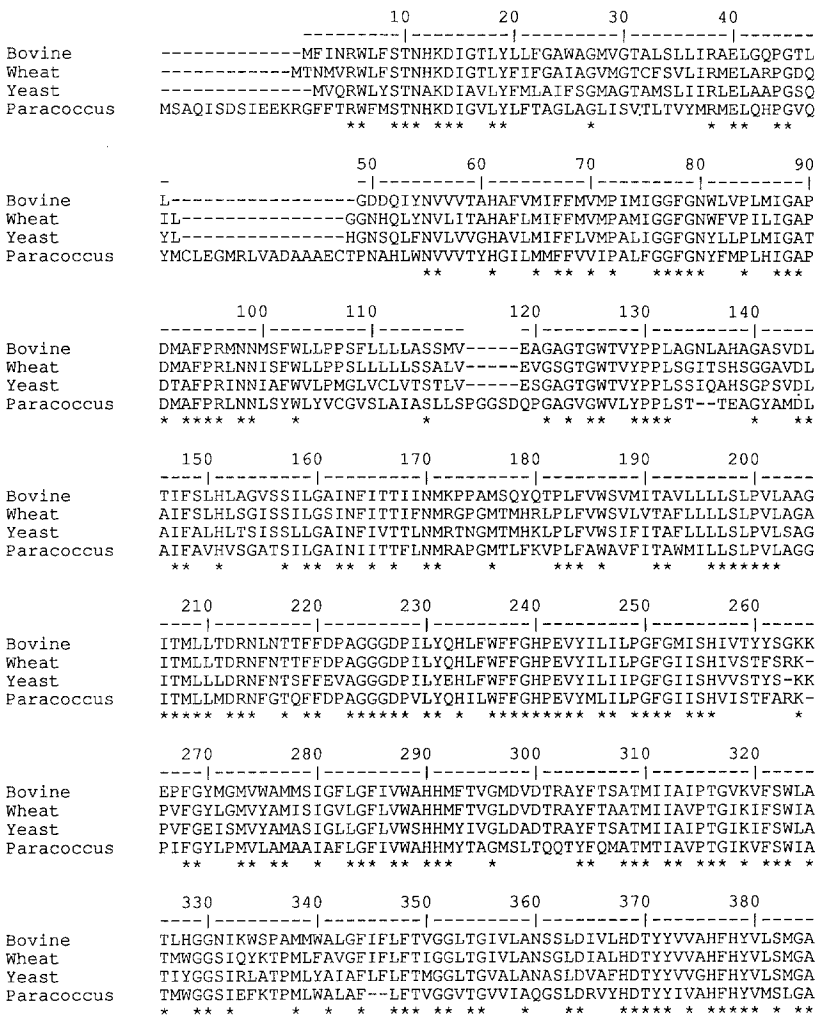


**Fig. 4.** Homologous alignments of DNA-deduced sequences of subunit I of cytochrome *c* oxidase from *Paracoccus denitrificans* (Haltia *et al.*, 1987) to sequences from bovine heart (Anderson *et al.*, 1981), wheat (Bonen *et al.*, 1987), and yeast (Bonitz *et al.*, 1980). The numbers refer to the positions in the bovine heart sequence, and the stars highlight the invariant amino acids in this comparison.

in the *Paracoccus* subunit I. Side reactions are more likely to occur in the bacterial biosynthesis than in mitochondria. Besides positioning of an N-terminal pyroglutamate as in the subunit II (Steinrücke *et al.*, 1987) and other protein sequences of this bacterium questions remain with respect to the C-terminus which has not been thoroughly investigated. Nevertheless the

```
                 390        400        410        420        430        440
                ----|---------|---------|---------|---------|---------|-----
Bovine          VFAIMGGFVHWFPLFSGYTLNDTWAKIHFAIMFVGVNMTFFPQHFLGLSGMPRRYSDYPD
Wheat           VFALFAGFYYWVGKIFGWTYPETLGQIHFWITFFGVNLTFFPMHFLGLSGMPRRIPDYPD
Yeast           IFSLFAGYYYWSPQILGLNYNEKLAQIQFWLIFIGANVIFFPMHFLGINGMPRRIPDYPD
Paracoccus      LFAIFAGTYYWIGKMSGRQYPEWAGQLHFWMMFIGSNLIFFPQHFLGRQGMPRRYIDYPV
                 *    *   *     *           *   * * *  *** **** *****  ***

                 450        460        470        480              490
                ----|---------|---------|---------|-------       --|---------
Bovine          AYTMWNTISSMGSFISLTAVMLMVFIIWEAFASKREVLTVDL------TTTNLEWLNGCP
Wheat           AYAGWNALSSFGSYISVVGIRRFFVVVAITSSSGKNQKCAESPWAVEQNPTTLEWLVQSP
Yeast           AFAGWNYVASIGSFIATLSLFLFIYILYDQLVNNKSVIYAKA------PSSSIEFLLTSP
Paracoccus      EFSYWNNISSIGAYISFASFLFFIGIVFYTLFAGKPVNVPNY---WNEHADTLEWTLPSP
                    **   * *  *                                    *     *

               500        510        520
                 |---------|---------|--
Bovine          PPYHTFEEPTYVNLK
Wheat           PAFHTFGELPAVKETKS
Yeast           PAVHSFNTPAVQS
Paracoccus      PPEHTFETLPKPEDWDRAQAHR
                *  * *
```

**Fig. 4.** Continued.

general framework of subunit I sequences may be considered sound on the basis of the one-protein-derived sequence and the analytical work performed with the enzymes or isolated polypeptides.

On this basis then, the hydroplot of these chains has been interpreted to show 8–12 membrane penetrating sections (Welinder and Mikkelsen, 1983; Wikström and Saraste, 1984). As deduced from protein chemical evidence recently obtained after treatment with different proteases with bovine heart cytochrome *c* oxidase right-side-out reconstituted in phosphatidylcholine vesicles (Willems, 1990), the general orientation as described by Wikström and Saraste (1984) is correct; however, the number of membrane penetrations is more likely to be 10, and the N- and C-terminus may be oriented outward, a structure which can also more favorably be packed into the $M_1$ domain of the enzyme (Bisson and Montecucco, 1985) according to the available 3d data (Deatherage *et al.*, 1982). According to the highly conserved subunit I structure, this conformation may be assumed also for the *Paraccocus* protein assembled in the plasma membrane.

We have not included more distantly related subunit I sequences as those obtained from the *E. coli* cytochrome *o* (Anraku and Gennis, 1987) and the *Thermus thermophilus* $caa_3$ oxidase (Mather *et al.*, 1988). These species do not belong to the purple bacteria $\alpha$ group from which mitochondria have originated. The phylogenetic distance in the 16s rRNA systematics leads back to association coefficients *s* of 0.3 and lower and falls below the $O_2$ horizon at about 0.5*s* (0.02% of present atmospheric level) (Fox *et al.*, 1980). The distance calculated from the available protein and gene data is in agreement with these 16s rRNA data.

It is therefore no surprise that both proteins besides exhibiting regions

of strong similarity—which probably is a true homology—also possess structural segments which are not common to the subunit I sequences aligned above and thus do not allow a simple transformation of data at least from a structural point of view. The above aligned subunit I sequences then contain a series of conserved residues, among them 12 histidines some of which are in positions that are likely to be involved in complexing the canonical metal centers of hemes *a*, $a_3$, copper B, and possibly the third copper.

As to speculation of the assignment of these residues and the molecules' folding pattern to these prosthetic groups (and their relation to proximal and distal histidines of hemoglobins) (Wikström and Saraste, 1984), we would like to await the X-ray structure of the oxidase.

As outlined above, the *Paracoccus* oxidase subunit II has been characterized by a cooperative protein chemical and genetic effort (Steinrücke *et al.*, 1987). In this case, therefore, there is a safe alignment to the mitochondrial subunit II sequences, among them the bovine protein, which was one of the first protein sequences obtained from cytochrome *c* oxidase (Steffens and Buse, 1976). The wheat (Covello and Gray, 1989) protein has not been characterized protein chemically in detail (Fig. 5). The yeast sequence (Coruzzi and Tzagoloff, 1979) has a 15-residue leader peptide (Pratje *et al.*, 1983) and starts with Asp-Val-Pro- (Fig. 5). Unfortunately this finding is not considered even in the Protein Information Resource (1989) database. Also the original papers on these DNA-derived sequences had not fully recognized the existing alteration of the mitochondrial genetic code. Thus, in the yeast sequence, ATA coding for positions 63, 115, and 167 had been translated as isoleucine instead of methionine. The exact status of the proteins is again hypothetical. However, also in subunit II the alignment is altogether convincing and several common features, which have been used for a functional characterization, emerge. The protein has an N-terminal two-fold membrane sequence, indicating an amphilic character with N-terminus and a large hydrophilic domain extending to the C-terminus at the outer side of the mitochondrial or plasma membrane, respectively. Among the invariant residues two histidines 161 and 204 and two cysteines 196 and 200 (bovine numbering) and a methionine in 207 are considered important (Buse *et al.*, 1978). A construction with two histidines and two sulfur-containing amino acids can therefore invariably be deduced as a possible copper-complexing array in agreement with spectroscopic data and has been claimed for the properties associated with copper A (Craig *et al.*, 1988). Cystine 200 in the wheat sequence (bovine numbering, Fig. 5) deserves a special comment. In the DNA sequence an arginine is coded in this position; however, the CGT codon is changed to UGU by the mysterious "editing" of the mRNA (Covello and Gray, 1989), suggesting that cysteine will stand in the protein as expected.

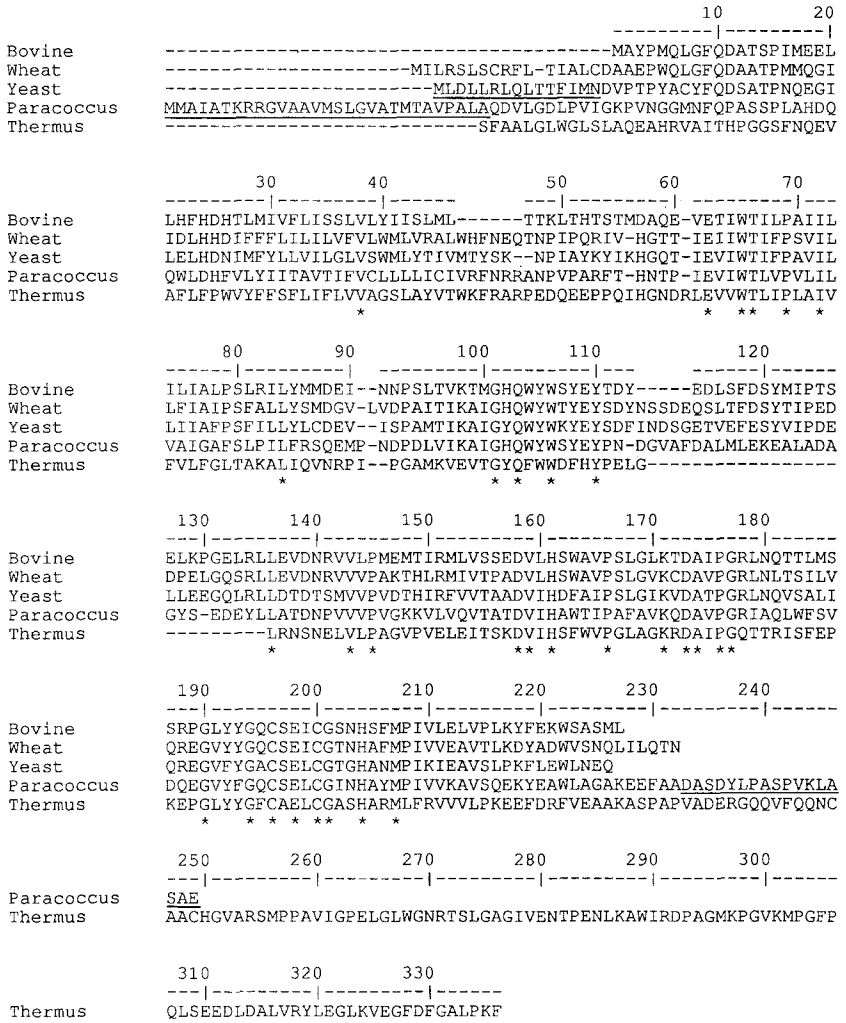


**Fig. 5.** Homologous alignment of amino acid sequences of subunit II of cytochrome *c* oxidase from *Paracoccus denitrificans* (Steinrücke *et al.*, 1987) to sequences from bovine heart (Steffens and Buse, 1979), wheat (Covello and Gray, 1988), yeast (Coruzzi and Tzagoloff, 1979), and of the 35-kDa subunit B of the $caa_3$-type terminal oxidase from *Thermus thermophilus* (Buse *et al.*, 1989; Mather *et al.*, 1990). The stars highlight the invariant amino acids in this comparison. Solid lines identify the amino acids which are removed by post-translational processing in the *Paracoccus* (Steinrücke *et al.*, 1987) and yeast (Pratje *et al.*, 1983) proteins. Numbers refer to the bovine sequence.

The above alignment of mitochondrial and the more closely related *Paracaccus* sequence does not limit the invariant histidines to two; this is, however, the case if the subunit II homolog sequence of the 35-kDa subunit of the *Thermus* $caa_3$-oxidase (Buse *et al.*, 1989; Mather *et al.*, 1990) is included (Fig. 5). Though this is a different molecule, this information will probably exclude the possibility of complexing additional redox active metal centers in the chain, which then are positioned in subunit I. Also the *Trypanosoma* sequence (Hensgens *et al.*, 1984) has been used for this argumentation. However, its DNA-derived structure is not in agreement with the systematic position to be expected for this species and thus indicates that something must be different with this enzyme.

Another completely invariant feature among the *Paracoccus* and mitochondrial sequences is the –Trp–Tyr–Trp–X–Tyr–Glu–Tyr– cluster. This has been suggested to be involved in electron conduction between copper A and an accepting centre in subunit I (Steffens and Buse, 1979). The authors have even speculated about the possibility of coupling of $e^-$ and $H^+$ translocation at this site via the tyrosine half-chinone interacting with an invariant carboxyl (Buse *et al.*, 1984) (Glu in this sequence or heme *a* propionyl). Such speculation has to await the X-ray data, which in the case of the bacterial photoreaction center tell us that even very fast $e^-$-conduction (tunnelling) via protein structures seems not to require a special (aromatic) arrangement (Huber, 1989). Rapid electron equilibration between an aromatically sandwiched two-copper couple may still be possible at this site.

Though clearly homologous, the *Paracoccus* protein deviates from the mitochondrial species in some characteristic positions which describe a different high-affinity binding to a *c* cytochrome. Among these are Lys 120, Tyr 134, Ala 137, Leu 151, and Lys 212 where charges have disappeared or are inverted. Others, as for instance Asp 158, close to the putative copper ligand His 161 are preserved.

Comparison of the sequence alignments of subunits I and II and the *c*-cytochromes of these species show that subunits I and cytochromes *c* both exhibit a similarly conserved structure with about 60% identity among the animal, plant, and fungal kingdoms and about 50% of these to the *Paracoccus* subunit I and cytochrome $c_2$ sequences. Subunit II giving about 44% and 33%, respectively, is not that much conserved. This is surprising if there is a strict cytochrome *c* → subunit II → subunit I arrangement of $e^-$ flow. An explanation may be that the hemes pose additional structural constraints on both cytochromes.

Sequence and functional comparison between the mitochondrial subunits I and II and the corresponding *Paracoccus* sequences as depicted above, gave the first firm evidence for these two proteins as functional, catalytic subunits in respiratory complexes IV (Steffens *et al.*, 1983). In addition, it

```
                                  *   **  *   * * *   *
Azurin                            YEFFCS--CFPGHGAGMVGKV

Subunit II                        YFGQCSELCGINH-AYMPIVV
Cytochrome c oxidase

N2O-Reductase                     YY--CSWFCHALH-MEMVGRM
                                  *    **  *   *   *
```

**Fig. 6.** Alignment of partial sequences—copper binding motive—of azurin from *Pseudomonas fluorescens* (Adman *et al.*, 1979), subunit II of cytochrome *c* oxidase from *Paracoccus denitrificans* (Steinrücke *et al.*, 1987), and $N_2O$-reductase from *Pseudomonas stutzeri* (Viebrock and Zumft, 1988).

demonstrated the prokaryotic origin of $aa_3$ oxidases. This being established, it is not surprising to find connections between the terminal segments of bacterial electron transport chains (e.g., of bacterial photosynthesis or photophosphorylation) and electron-conducting units of cytochrome *c* oxidase. These have been found for subunit II (Buse *et al.*, 1978). Figure 6 shows an alignment of partial sequences of the *Paracoccus* subunit II with the small blue copper protein azurin from *Pseudomonas fluorescens* (Adman *et al.*, 1978) and a C-terminal sequence of nitrous oxide reductase from *Rhodopseudomonas stutzeri* (Viebrock and Zumft, 1988). In both cases a sufficient scoring of homology can hardly be derived solely by computation but needs the additional assignment as "copper protein" to identify and align the two-histidine–two-sulfur amino acid copper binding site. The proteins are clearly different in function and structure but still contain a certain motive which may indicate a remote phylogenetic relationship. No such relation has yet been found for subunit I or parts thereof.

## Cytochrome Oxidase and the Growing $O_2$ Atmosphere

In the natural system of prokaryotes the photosynthetic purple bacteria and their nonphotosynthetic relatives are divided in four subgroups, $\alpha$, $\beta$, $\gamma$, $\delta$. According to the 16s rRNA phylogeny, their common origin reaches back beyond the earth $O_2$ horizon (Fox *et al.*, 1980). The $O_2$ atmosphere started at about $-2$ billion years after exhaustion of the soluble marine $Fe^{II}$ and formation of the well-determined $Fe_3O_4$ deposits (Levine, 1988). This leads to the conclusion that "it is clear that aerobic respiration has arisen many times; in the purple bacterial group, in particular, there are three clear examples of it and perhaps many more. Although the evolution of aerobic metabolism is not always associated with photosynthetic phenotypes, initially it often is" (Fox *et al.*, 1980).

*Paracoccus denitrificans* belongs to the purple bacteria $\alpha$ group (Stackebrandt, 1986). This group is also the source of mitochondria (Yang *et al.*, 1985).

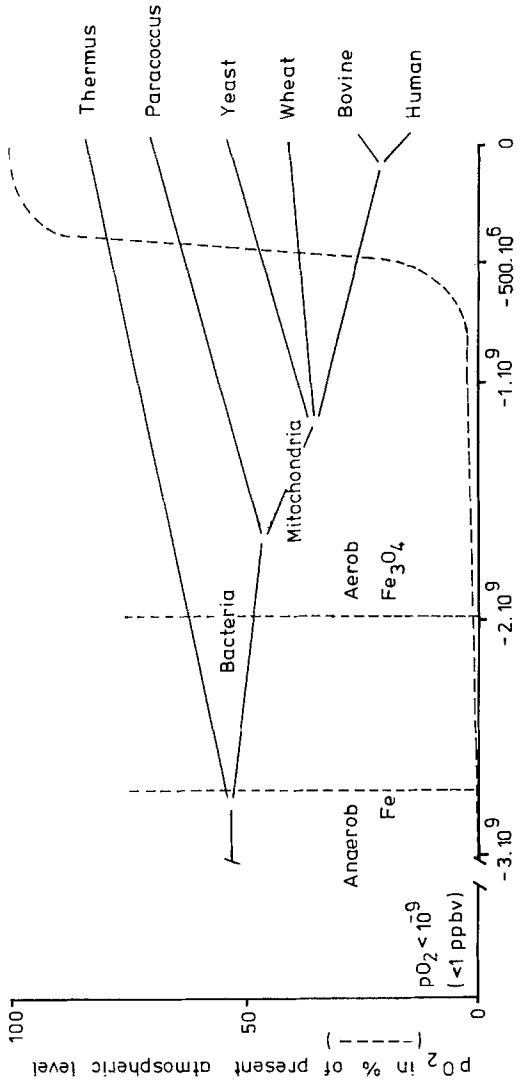


**Fig. 7.** Phylogenetic tree based on amino acid sequences of subunit I of cytochrome *c* oxidase from bovine heart (Anderson *et al.*, 1982; Hensel and Buse, 1990), wheat (Bonen *et al.*, 1987), yeast (Bonitz *et al.*, 1980), *Paracoccus denitrificans* (Raitio *et al.*, 1987), and *Thermus thermophilus*, partial sequence (Fee *et al.*, 1988), arranged in the growing $O_2$ horizon (Levine, 1988).

The symbiotic event is dated back at about $-1.5$ billion years or less (Cavalier-Smith, 1987) and thus falls into the growing $O_2$ atmosphere. Cytochrome *c* oxidase of *Paracoccus denitrificans*(Fig. 7) then is a descendant of the same invention of $O_2$ respiration as mitochondria. Other $O_2$-consuming bacterial oxidases as, for instance, cytochrome *o* of *E. coli* and the $caa_3$ oxidase of *Thermus thermophilus* or the $PS_3$ enzyme, do not fall into this continuum and represent independent developments toward the energy-conserving $O_2$ respiration.

Though there is obviously a common genetic substrate seen in the sequences of the heme and copper-binding subunits which reaches back to the origins of eubacteria (Buse *et al.*, 1989), the respiratory use of $O_2$ in those enzymes is a convergent development. Structural similarity then cannot simply be interpreted as homology.

The idea of horizontal gene transfer has sometimes been used to explain related functions in apparently separated systematic categories of prokaryotes. The available evidence, however, is not in favour of this idea. Independent data obtained from cytochrome *c* (Dickerson *et al.*, 1976), ferredoxin (Tanaka *et al.*, 1971), and the 5s rRNA and DNA/RNA hybridization are in line with 16s rRNA data (Fox *et al.*, 1980). This is also the case for sequences of terminal oxidases from prokaryotes themselves. As shown in Fig. 7, *Paracoccus denitrificans* is relatively close to mitochondria while the *Thermus* 70-KDa subunit represents a very remote relative of the same genetic material reaching back to the origin of eubacteria. Again the relative distances are in agreement with the 16s rRNA data. The primary function and the origin of the common genetic substrates, cooperating copper, and heme proteins, is not known. Photosynthetic and non-photosynthetic electron-transport chains of prokaryotes, however, provide examples of small blue copper proteins and *c* cytochromes, whose genes may have been fused—as in *Thermophilus* (Buse *et al.*, 1989)—and then duplicated to give the 2 copper/2 heme canonical catalytic center of cytochrome oxidase.

The functional step of $O_2$ respiration from purple bacteria to mitochondria shows that the most successful use of oxidative phosphorylation has not been made by the prokaryotes which invented this bioenergetic hit but by the "urkaryote" with its superior genetic capabilities: "managers dominate inventors."

## Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft Bu 463/1.

## References


Adman, E. T., Stenkamp, R. E., Sieker, L. C., and Jensen, L. H. (1978). *J. Mol. Biol.* **123**, 35–47.

Altmann, R. (1890). In *Die Elementarorganismen und ihre Beziehung zu den Zellen*, Veit, Leipzig.

Ambler, R. P., Meyer, T. E., Kamen, M. D., Schickman, S. A., and Sawyez, L. (1981). *J. Mol. Biol.* **147**, 351–356.

Anderson, S., Bankier, A. T., Barrell, B. G., De Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981). *Nature (London)* **290**, 457–465.

Anderson, S., De Bruijn, M. H. L., Coulson, A. R., Eperon, I. C., Sanger, F., and Young, I. G. (1982). *J. Mol. Biol.* **156**, 683–718.

Anraku, Y., and Gennis, R. B. (1989). *Trends Biochem. Sci.* **12**, 262–266.

Azzi, A., Bill, K., Bolli, R., Casey, R. P., Nalecz, K. A., and O'Shea, P. (1985). In *Structure and Properties of Cell Membranes* (Benga, G., ed.), Vol. II, CRC Press, pp. 105–138.

Berry, E. A., and Trumpower, B. L. (1985). *J. Biol. Chem.* **260**, 2458–2467.

Bisson, R., and Montecucco, C. (1985). *J. Inorg. Biochem.* **23**, 177–183.

Bonen, L., Boer, P. H., McIntosh, J. E., and Gray, M. W. (1987). *Nucleic Acids Res.* **15**, 6734.

Bonitz, S. G., Coruzzi, G., Thalenfeld, B. E., Tzagoloff, A., and Macino, G. (1980). *J. Biol. Chem.* **255**, 12927–12941.

Brunori, M., Colosimo, A., Rainoni, G., Wilson, M. T., and Antonini, E. (1979). *J. Biol. Chem.* **254**, 10769–10775.

Buse, G., Steffens, G. J., and Steffens, G. C. M. (1978). *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1011–1013.

Buse, G., Steffens, G. C. M., and Meinecke, L. (1983). In *Structure and Function of Membrane Proteins* (Quagliariello, E., and Palmieri, F., eds.), Elsevier Science Publishers, pp. 131–138.

Buse, G., Biewald, R., Raabe, G. and Fleischhauer, J. (1984). *3rd EBEC Reprints*, 119–120.

Buse, G., Meinecke, L., and Bruch, B. (1985). *J. Inorg. Biochem.* **23**, 149–153.

Buse, G., Steffens, G. C. M., Biewald, R., Bruch, B., and Hensel, S. (1987). In *Cytochrome Systems* (Papa, S., Chance, B., and Ernster, L., eds.), Plenum Press, New York, pp. 791–793.

Buse, G., Hensel, S., and Fee, J. A. (1989). *Eur. J. Biochem.* **181**, 261–268.

Cavalier-Smith, T. (1987). *Ann. N.Y. Acad. Sci.* **503**, 17–54.

Coruzzi, G., and Tzagoloff, A. (1979). *J. Biol. Chem.* **254**, 9324–9330.

Covello, P. S., and Gray, M. W. (1989). *Nature (London)* **341**, 662–665.

Craig, T. M., Scholes, C. P., and Chan, S. I. (1988). *J. Biol. Chem.* **263**, 8420–8429.

Deatherage, J. F., Henderson, R., and Capaldi, R. A. (1982). *J. Mol. Biol.* **158**, 500–514.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1985). *Nature (London)* **318**, 618–624.

Dickerson, R. E., Timkovich, R., and Almassy, R. J. (1976). *J. Mol. Biol.* **100**, 473–491.

Einarsdóttir, O., and Caughey, W. S. (1984). *Biochem. Biophys. Res. Commun.* **124**, 836–842.

Einarsdóttir, O., and Caughey, W. S. (1985). *Biochem. Biophys. Res. Commun.* **129**, 840–847.

Fee, J. A., Mather, M. W., Springer, P., Hensel, S., and Buse, G. (1988). *Cytochrome Oxidase: Structure, Function, and Physiopathology* (Brunori, M., and Chance, B., eds.), *Ann. N. Y. Acad. Sci.* **550**, 33–38.

Fox, G. E., Stackebrandt, E., Hespell, R. B., Gibson, J., Maniloff, J., Dyer, T. A., Wolfe, R. S., Balch, W. E., Tanner, R. S., Magum, L. J., Zablen, L. B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B. J., Stahl, D. A., Luehrsen, K. R., Chen, K. N., and Woese, C. R. (1980). *Science* **209**, 457–463.

Fukumori, Y., Nakayama, K., and Yamanaka, T., (1985). *J. Biochem.* **98**, 493–499.

Haltia, T., Puustinen, A., and Finel, M. (1988). *Eur. J. Biochem.* **172**, 543–546.

Hensel, S., and Buse, G. (1990). *Biol. Chem. Hoppe-Seyler* **371**, 411–422.

Hensgens, L. A. M., Brakenhoff, J., De Vries, B. F., Sloof, P., Tromp, M. C., Van Boom, J. H., and Benne, R. (1984). *Nucleic Acids Res.* **12**, 7327–7344.

Holm, L., Saraste, M., and Wikström, M. (1987). *EMBO J.* **6**, 2819–2823.

Huber, R. (1989). *EMBO J.* **8**, 2125–2147.

John, P., and Whatley, F. R. (1977). *Biochim. Biophys. Acta* **463**, 129–153.
Jukes, T. H. (1990). *J. Mol. Evol.* **30**, 1–2.
Kroneck, P. M. H., Antholine, W. A., Riester, J., and Zumft, W. G. (1988). *FEBS Lett.* **242**, 70–74.
Kyte, J., and Doolittle, R. F. (1982). *J. Mol. Biol.* **157**, 105–132.
Levine, S. (1988). In *Oxidases and Related Redox Systems* (King, R. E., Mason, H. S., and Morrison, M., eds.), Vol. 274, Alan R. Liss, Inc., New York, pp. 111–126.
Ludwig, B., and Schatz, G. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 196–200.
Ludwig, B., Grabo, M., Gregor, I., Lustig, A., Regenass, M., and Rosenbusch, J. P. (1982). *J. Biol. Chem.* **257**, 5576–5578.
Mather, M. W., Springer, P., and Fee, J. A. (1990). In *The Molecular Basis of Bacterial Metabolism* (Hauska, G. and Thauer, R., eds.) 41st Mosbach Colloquium, Springer Verlag, pp. 94–104.
Merle, P., and Kadenbach, B. (1980). *Eur. J. Biochem.* **105**, 499–507.
Pratje, E., Mannhaupt, G., Michaelis, G., and Beyreuther, K. (1983). *EMBO J.* **2**, 1049–1054.
Protein Information Resource (PIR) databank, Georgetown University U.S. Release 23, 1989.
Raitio, M., Jalli, T., and Saraste, M. (1987). *EMBO J.* **6**, 2825–2833.
Schroedl, N. A., and Hartzell, C. R. (1977). *Biochemistry* **16**, 4916–4965.
Seelig, A., Ludwig, B., Seelig, J., and Schatz, G. (1981). *Biochim. Biophys. Acta* **636**, 162–167.
Sevarino, K. A., and Poyton, R. O. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 142–146.
Sone, N., and Yanagita, Y. (1982). *Biochim. Biophys. Acta* **682**, 216–226.
Stackebrandt, E. (1986). *Forum Mikrobiol.* **5**, 255–260.
Steffens, G., and Buse, G. (1976). *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1125–1137.
Steffens, G. C. M., and Buse, G. (1990). 6th EBEC Reports.
Steffens, G. C. M., Buse, G., Oppliger, W., and Ludwig, B. (1983). *Biochem. Biophys. Res. Commun.* **116**, 335–340.
Steffens, G. C. M., Biewald, R., and Buse, G. (1987). *Eur. J. Biochem.* **164**, 295–300.
Steffens, G. C. M., Pascual, E., and Buse, G. (1990). *J. Chromatogr.* **527**, 297–299.
Steinrücke, P., Steffens, G. C. M., Panskus, G., Buse, G., and Ludwig, B. (1987). *Eur. J. Biochem.* **167**, 431–439.
Tanaka, M., Haniu, M., Matsueda, G., and Yasunobu, K. T. (1971). *J. Biol. Chem.* **246**, 3953–3960.
Van Gelder, B. F., and Beinert, H. (1969). *Biochim. Biophys. Acta* **189**, 1–24.
Verheul, E. A. M., Draijer, J. W., Dentener, I. K., and Muijsers, A. O. (1981). *Eur. J. Biochem.* **119**, 401–408.
Viebrock, A., and Zumft, W. G. (1988). *J. Bacteriol.* **170**, 4658–4668.
Von Heijne, G. (1983). *Eur. J. Biochem.* **133**, 17–21.
Welinder, K. G., and Mikkelsen, L. (1983). *FEBS Lett.* **157**, 233–239.
Wikström, M., and Saraste, M. (1984). In *Bioenergetics* (Ernster, L., ed.), Elsevier Science Publishers B.V., Amsterdam, pp. 49–94.
Willems, U. (1990). Ph.D. thesis, RWTH Aachen.
Woese, C. R. (1987). *Microbiol. Rev.* **51**, 221–271.
Woese, C. R., Gibson, J., and Fox, E. (1980). *Nature (London)* **283**, 212–214.
Yang, D., Oyaizu, Y., Oyaizu, H., Olsen, G. J., and Woese, C. R. (1985). *Proc. Natl. Acad. Sci. USA* **82**, 4443–4447.
Yoshida, T., Lorence, R. M., Choc, M. G., Tarr, G. E., Findling, K. L., and Fee, J. A. (1984). *J. Biol. Chem.* **259**, 112–123.